

AD_____

Award Number: W81XWH-11-1-0360

TITLE: Selective Gene Regulation by Androgen Receptor in Prostate Cancer

PRINCIPAL INVESTIGATOR: Pia Bagamasbad, Ph.D.

CONTRACTING ORGANIZATION: University of Michigan Medical School
Ann Arbor, MI 48109-5618

REPORT DATE: October 2013

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE October 2013		2. REPORT TYPE Annual Summary		3. DATES COVERED 30 September 2012 – September 2013	
4. TITLE AND SUBTITLE Selective Gene Regulation by Androgen Receptor in Prostate Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-11-1-0360	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Pia Bagamasbad, Ph.D. E-Mail: piab@umich.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Michigan Medical School Ann Arbor, MI 48109-5618				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Growth and development of the prostate is highly dependent on androgen, and aberrant androgen and androgen receptor (AR) signaling is the key driver in the pathology and progression of prostate cancer. Blocking androgen synthesis and inhibiting AR function is the first line of therapy for prostate cancer patients. Although this approach is initially effective in suppressing disease progression, castration resistant tumors eventually develop resulting from reactivation of AR activity. A novel approach to overcome resistance to therapy is to identify selective AR modulators (SARMs) that inhibit the expression of genes promoting tumor growth but enable the expression of genes for differentiation. We hypothesize that functionally distinct set of genes have different promoter signature marks that are recognized by AR. Re-testing and validation of several hits obtained from a high-throughput promoter-dependent compound screen of FDA approved drugs identified a lead compound that had a differential effect on AR promoter-element recognition in transient transfection assays, and in the expression of select AR target genes associated with proliferation and differentiation. Cell proliferation assays indicate that the compound is able to inhibit AR-dependent cell growth. Our discovery of this compound in an unbiased screen for SARMs provides proof-of-concept that drugs may be developed that differentially target promoter elements that distinguish pro-proliferation from pro-differentiation genes.					
15. SUBJECT TERMS Androgen receptor, antiandrogens, prostate cancer cells. high throughput drug screen, differential promoter activation and gene regulation					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	11	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	9
Reportable Outcomes.....	9
References.....	10
Appendices.....	n/a

SELECTIVE GENE REGULATION BY ANDROGEN RECEPTOR IN PROSTATE CANCER

INTRODUCTION

Androgens mediate their biological effects by binding to the androgen receptor (AR), which functions as a ligand inducible transcription factor. Growth and development of the prostate is highly dependent on androgen and aberrant androgen and AR signaling is the key driver in the pathology and progression of prostate cancer [1]. Thus, the first line of therapy for prostate cancer patients consists of blocking androgen synthesis (i.e., by abiraterone) and inhibiting AR function with antagonists (i.e., enzalutamide) [2]. Although this approach is initially effective in suppressing disease progression, castration resistant tumors eventually develop resulting from reactivation of AR signaling [1-4]. Androgen receptor reactivation can arise from AR amplification, mutation, and altered coregulator interactions, all of which have been shown to be mechanisms underlying recurrent AR activity and formation of castration-resistant prostate cancer (CRPC) [3]. Current prostate cancer therapies are circumvented in CRPC [3, 5] and there is an outstanding need to identify and develop anti-tumor drugs effective in conditions where AR is reactivated, overexpressed or mutated. In this project, our goal is to identify selective AR modulators (SARMs) that inhibit the expression of genes promoting tumor growth but enable the expression of genes for differentiation. This strategy may identify SARMs that confer selective gene regulation that can reduce resistance and may be useful as an adjuvant therapy against AR reactivation.

BODY

The grant outlined 3 tasks in the Statement of Work:

Task 1: Examine the biological effects of the mutant ARs using cell based assays and xenografts

Task 2: Compare the gene expression programs differentially regulated by the mutant ARs

Task 3: Identify molecules that alter AR activity in a promoter-specific manner.

Task 1 and Task 2 were initiated by Dr. E. Lapensee who left for a permanent position. The fellowship transferred to me in November 2012, and I primarily focused on Task 3 in order to validate and characterize primary hits from the promoter-dependent high-throughput screen, as described below.

Pilot screen. AR regulates gene transcription by directly binding to DNA or by protein-protein interactions. Hormone response elements bound by AR can be classified as canonical androgen response elements (cARE) composed of 6 bp inverted repeats shared with other steroid receptors or selective AREs (sARE), which are 6 bp direct repeats and selectively bind AR (Fig 1) [6, 7]

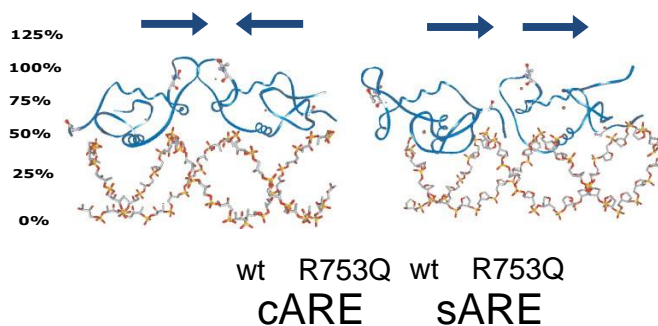


Fig. 1 – Consensus vs. selective AREs.

The AR DNA binding domain dimerizes in a head-to-head fashion on inverted repeats but head-to-tail on direct repeats (modeled from x-ray crystal data). The different configuration imposed by the DNA element exposes different AR surfaces to coregulators.

Studies have shown that allosteric changes in AR conformation may be influenced by the bound DNA sequences [8]. Similarly, gene recognition by AR also depends on promoter context, cell type and development or disease stage. Genome wide analysis of AR binding sites has shown that genes regulated by AR in CRPC are distinct from those in normal cells or early disease [9]. Mutant ARs can direct different gene regulatory programs and, in some cases, have gained preference for activating cAREs over sAREs [10-12]. Moreover, mutations in AR that eliminate binding to sAREs affect the reproductive system and result in reduced fertility in male mice suggesting that genes involved in differentiation rely more on sAREs [13]. Taken together this suggests that the sequence and context of AR binding has an important role in gene-specific transcriptional control. Further, compounds that interact with different AR domains may influence promoter choice and DNA-binding capacity. Based on this rationale, we developed a high-throughput screen to identify compounds with a differential effect on AR activity dependent on promoter elements. A similar approach succeeded in identifying glucocorticoid receptor modulators [14].

Differential AR activation was assessed using transfected fluorescent reporters driven by multimerized cAREs, sAREs or a PSA promoter in HeLa cells stably expressing AR (HeLa-A6). Screens were performed in saturating levels of the synthetic androgen R1881 to envision promoter activity rather than ligand competition, and optimized for maximal separation of activation vs. AR antagonist inhibition. The pilot screen included 2500 compounds in the Spectrum FDA-approved library and drugs used in NIH clinical trials. Of these compounds, 8% suppressed AR-driven promoters by >75%, and about 3/4 of those suppressed cAREs and sAREs similarly. As an indication of screen validity, known anti-androgens were identified in the screen. To view selectivity, compounds that suppressed one ARE by more than 75% but the other by less than 25% were tallied (Fig. 2). There were 22 cARE-selective compounds that strongly suppressed cARE and had minimal effect on sARE activity. Dose response assays identified 5 cARE-selective compounds with acceptable inhibition curves and potencies to proceed to secondary assays.

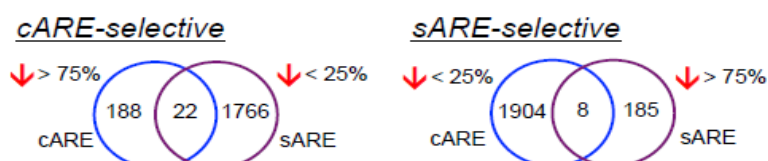
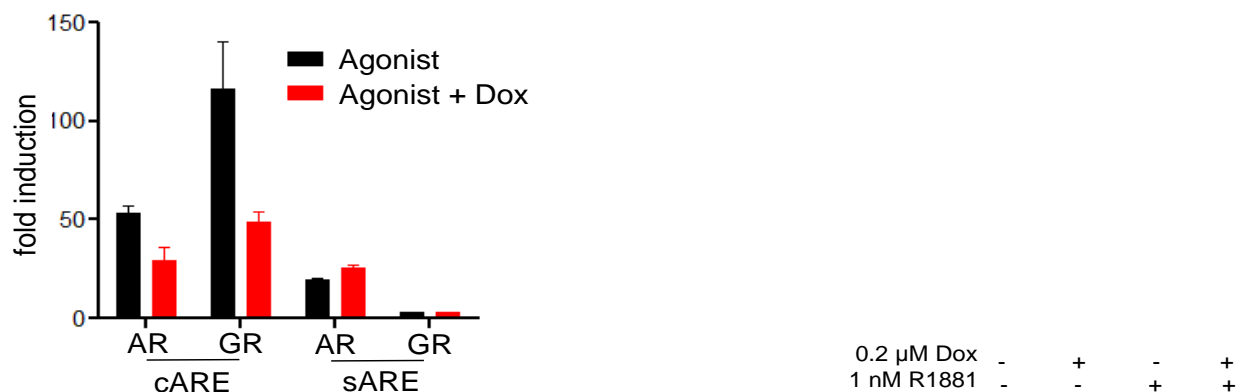


Fig 2. Venn diagrams show overlapping sets of drugs that strongly suppress one ARE type but have little effect on the other.

Validation of primary screen hits in transient transfection luciferase assays. Fresh powder samples of the 5 lead compounds were used to confirm compound activity in transfection assays using cARE, sARE and PSA promoters driving luciferase reporters. Transfection assays were done in robust and easily transfected HeLa-A6 and CV-1 fibroblasts, in a normal prostate epithelial cell line RWPE (AR null), and in several PCa cell lines that include LAPC4 (wt AR), LNCaP (promiscuous AR-T877A), VCaP (high levels of wt AR), and PC-3 (AR null). HeLa and HeLa-A6 cells were extremely sensitive to even very low doses of compounds. The PCa cell lines, LNCaP, VCaP and LAPC4, had very low transfection efficiencies. Androgen-induced



Doxorubicin is an anthracycline drug that is widely used as a chemotherapeutic agent with a wide spectrum of antitumor activity. Although dox remains one of the most effective chemotherapeutic agents, its mechanism of action is not fully understood. Dox is known to intercalate into DNA and disrupt topoisomerase II action resulting in DNA double strand breaks. This activates the DNA damage response (DDR) to repair the DNA break, and when repair is not successful, apoptosis is initiated [15]. Dox increases the expression of tumor suppressor genes p21 and p53 via unknown mechanisms. Dox also generates free radicals that can damage cell membranes, DNA and proteins which triggers the apoptotic pathway [16]. These actions of dox are regarded to be the underlying mechanisms for its anti-cancer activity. However, recent reports show that dox regulates transcription by enhancing nucleosome turnover or histone eviction at promoters and this occurs independent of its ability to cause DNA strand breaks and initiating DDR. [17, 18]. X-ray crystallography, biochemical data and computer modeling indicate that dox binds to DNA with some sequence selectivity with preferential binding to alternating purine-pyrimidine tracts and affinity affected by neighboring bases. Dox provides proof-of-concept that our transcription based screen detects differences between AREs that model promoter signatures differentially used by AR targets and genes involved in AR signaling.

Effect of compound on endogenous gene expression. To eliminate the possibility that any differential effect of dox on gene expression may be due to changes in AR expression or localization, we determined the effect of dox on AR mRNA, protein and nuclear localization in

LNCaP cells. Dox did not change AR mRNA expression (not shown) and androgen-induced protein levels (Fig 4A), and did not alter androgen- induced nuclear localization (Fig 4B).

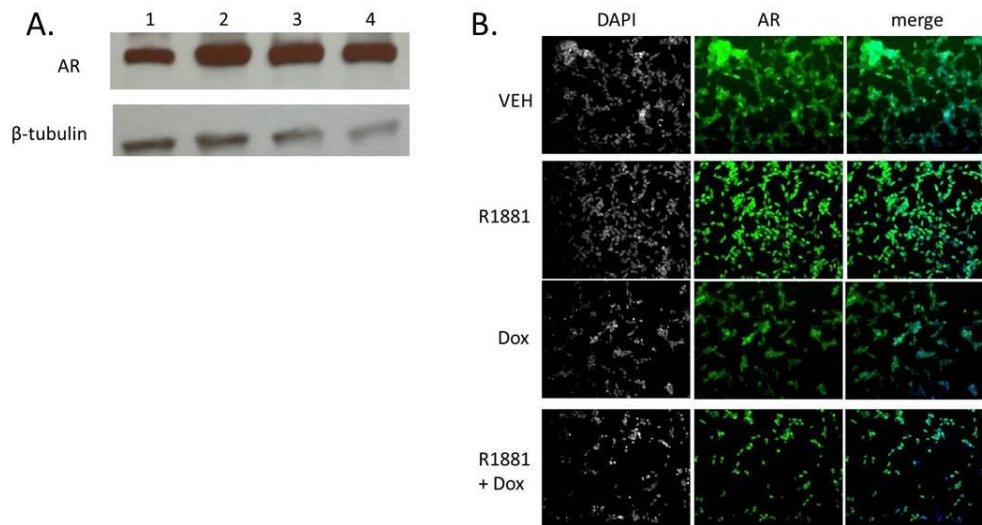


Fig. 3 – Dox does not affect AR expression. A. Western blot analysis for AR and β -tubulin (loading control) in LNCaP cells treated with 1) vehicle (VEH), 2) 10 nM R1881, 3) 0.1 μ M Dox + R1881, and 4) 0.3 μ M Dox + R1881 cells for 24h. B) Immunofluorescent staining of nucleus (DAPI) and AR in LNCaP cells treated with VEH, 10 nM R1881, 0.4 μ M Dox and R1881+ Dox.

To determine if the differential effect of dox occurs in natural promoters, particularly those that are active in normal vs cancer growth, we determined the effect of dox on select AR target genes in prostate cell lines. Since we were interested in inhibiting AR mediated cell proliferation, we looked at the effect of dox on the expression of AR target genes involved in cell cycle regulation. Cyclin dependent kinase 1 (CDK1) is an androgen regulated gene [19] that functions in the S phase of the cell cycle and promotes entry into mitosis [20]. Interestingly, CDK1 has been shown to phosphorylate AR to increase its stability [21]. The cell cycle regulator p21 is an androgen induced gene that functions as a tumor suppressor by inhibiting CDK activity [22]. In LNCaP cells (Fig 5A), dox had an anti-proliferative effect by inducing p21 expression and repressing androgen-induced expression of CDK1. Dox had varied effects on AR target genes associated with differentiation where it repressed expression of FKBP5 and AQ3, and induced expression of SGK1. For these genes, binding sites for dox or AR are unclear. However, sARE-like elements are essential for AR dependent regulation of SGK1 since male mice that cannot recognize sAREs fail to express SGK1 [13]. Gene expression was also examined in normal prostate epithelia using RWPE cells transduced with wt AR (RWPE-AR) to determine the effect of dox in a more normal background (Fig 5B). In RWPE-AR cells, dox effects were modest and mostly suppressive. In contrast, in AR null PCa cells (PC-3), dox strongly induced SGK1 and repressed CDK1 as in LNCaP but repressed p21 (Fig 5C). Taken together, dox exhibits gene- and cell-specific effects that may occur via AR as well as through AR-independent mechanisms. In addition, the magnitude of dox response is more pronounced in PCa than in normal prostate epithelia.

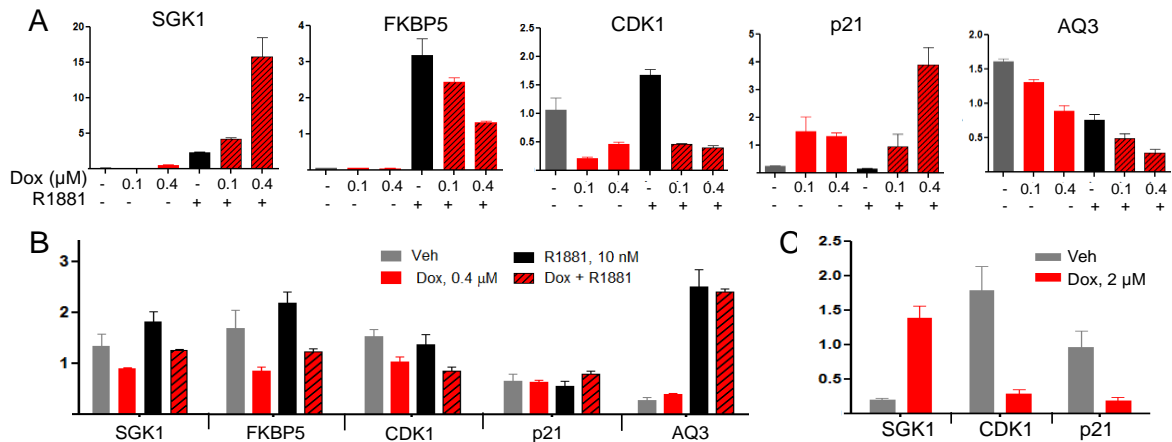


Fig. 5 – Dox effects are gene- and cell-specific. Endogenous gene expression was assayed by qRT-PCR of RNA from: **A) LNCaP cells, B) RWPE-AR cells and C) PC-3 cells.** Cells were plated in 2.5% CSS for 3 days, and then treated for 24 hrs with 0.1 or 0.4 μM dox alone or with 10 nM R1881. qPCRs were normalized to GAPDH.

To determine if the differential effect of dox on gene expression occurs primarily at the level of transcription, we measured the levels of nascent RNA transcripts. This was accomplished by labeling newly transcribed RNA with bromouridine (BrU), capturing the BrU-containing RNA with anti-BrU antibodies conjugated to magnetic beads and measuring nascent transcript levels by qPCR. We treated LNCaP cells with R1881 and dox for 24 hours and labeled with BrU for one hour before collecting cells for RTqPCR. Results showed that nascent transcript levels at

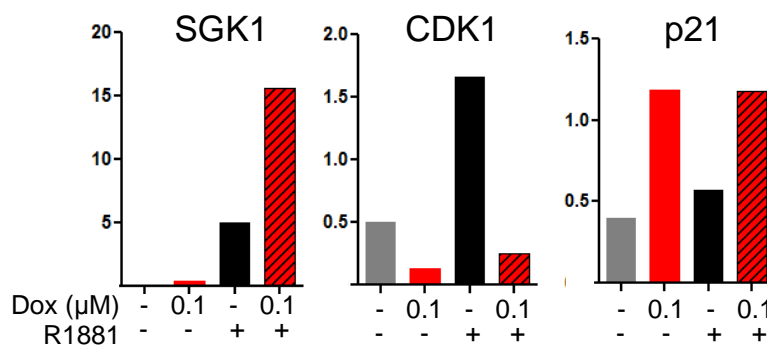


Fig. 6 – Dox selectively targets genes at an early step in transcription. LNCaP cells were starved in 2.5% CSS 3 days before treatment for 24 hrs with 0.1 μM dox, 1 nM R1881 or both. 2 mM Bru was added for 1 hr, Bru-RNA isolated and qRT-PCR performed.

Effect of compound on cell growth. To determine the effect of dox on AR-driven biology, we measured the effect of dox on cell viability and growth rate by MTT assay. At low dose, dox did not alter LNCaP growth but blocked AR-induced proliferation (Fig 7). These results fulfill one of the objectives of the overall cancer-cancer research.

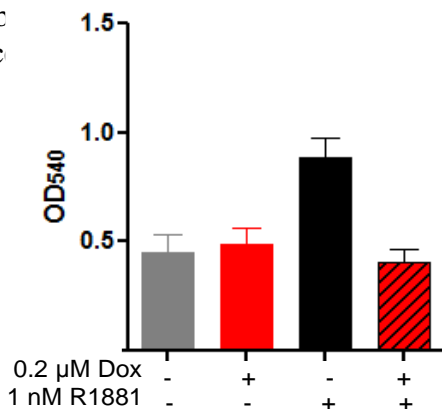


Fig. 7 – Dox inhibits AR-induced growth of LNCaP cells. Cells were plated in 2.5% CSS 3 days prior to adding dox +/- R1881 for 5 days. Growth is assessed by MTT assay. Dox has no effect alone but blocks AR-dependent proliferation.

KEY RESEARCH ACCOMPLISHMENTS

- We have re-tested the lead compounds from a pilot high-throughput screen designed to identify selective AR antagonists. Re-testing and compound validation was done using non-prostate, benign prostate and prostate cancer cell lines.
- We determined the receptor specificity and dependence of the lead compound that showed consistent activity in re-testing and validation assays.
- We have identified a lead compound, doxorubicin, that showed promoter selective effects by suppression of cARE but not sARE activity in luciferase-reporter assays and by differential regulation of the expression of AR target genes associated with proliferation and differentiation.
- We have shown by gene expression analysis that doxorubicin affects the transcription of AR target genes in a gene and cell-context specific manner, with a more pronounced transcriptional effect observed in PCa than normal prostate epithelia.
- We have shown that doxorubicin inhibits the AR-dependent growth of PCa cells.

REPORTABLE OUTCOMES

CONCLUSION

A novel approach to overcome resistance to therapy is to identify selective AR modulators (SARMs) that inhibit the expression of genes promoting tumor growth but enable the expression of genes for differentiation. We hypothesize that functionally distinct set of genes have different promoter signature marks that are recognized by AR. A high-throughput multiplexed promoter-dependent compound screen identified a lead compound that provides proof-of-concept that promoter elements distinguish pro-proliferation from pro-differentiation genes and that these compounds can affect AR element recognition and AR target gene expression in a cell-dependent manner.

REFERENCES

1. Scher, H.I. and C.L. Sawyers, Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. *J Clin Oncol*, 2005. 23(32): p. 8253-61.
2. Haendler, B. and A. Cleve, Recent developments in antiandrogens and selective androgen receptor modulators. *Mol Cell Endocrinol*, 2012. 352(1-2): p. 79-91.
3. Knudsen, K.E. and T.M. Penning, Partners in crime: deregulation of AR activity and androgen synthesis in prostate cancer. *Trends Endocrinol Metab*, 2010. 21(5): p. 315-24.
4. Sadar, M.D., Small molecule inhibitors targeting the "achilles' heel" of androgen receptor activity. *Cancer Res*, 2011. 71(4): p. 1208-13.
5. Chen, Y., C.L. Sawyers, and H.I. Scher, Targeting the androgen receptor pathway in prostate cancer. *Curr Opin Pharmacol*, 2008. 8(4): p. 440-8.
6. Claessens, F., P. Alen, A. Devos, B. Peeters, G. Verhoeven, and W. Rombauts, The androgen-specific probasin response element 2 interacts differentially with androgen and glucocorticoid receptors. *J Biol Chem*, 1996. 271(32): p. 19013-6.
7. Schoenmakers, E., P. Alen, G. Verrijdt, B. Peeters, G. Verhoeven, W. Rombauts, and F. Claessens, Differential DNA binding by the androgen and glucocorticoid receptors involves the second Zn-finger and a C-terminal extension of the DNA-binding domains. *Biochem J*, 1999. 341 (Pt 3): p. 515-21.
8. Geserick, C., H.A. Meyer, and B. Haendler, The role of DNA response elements as allosteric modulators of steroid receptor function. *Mol Cell Endocrinol*, 2005. 236(1-2): p. 1-7.
9. Wang, Q., W. Li, Y. Zhang, X. Yuan, K. Xu, J. Yu, Z. Chen, R. Beroukhi, H. Wang, M. Lupien, T. Wu, M.M. Regan, C.A. Meyer, J.S. Carroll, A.K. Manrai, O.A. Janne, S.P. Balk, R. Mehra, B. Han, A.M. Chinnaiyan, M.A. Rubin, L. True, M. Fiorentino, C. Fiore, M. Loda, P.W. Kantoff, X.S. Liu, and M. Brown, Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. *Cell*, 2009. 138(2): p. 245-56.
10. Hay, C.W. and I.J. McEwan, The impact of point mutations in the human androgen receptor: classification of mutations on the basis of transcriptional activity. *PLoS One*, 2012. 7(3): p. e32514.
11. O'Mahony, O.A., M.P. Steinkamp, M.A. Albertelli, M. Brogley, H. Rehman, and D.M. Robins, Profiling human androgen receptor mutations reveals treatment effects in a mouse model of prostate cancer. *Mol Cancer Res*, 2008. 6(11): p. 1691-701.
12. Thompson, V.C., T.K. Day, T. Bianco-Miotto, L.A. Selth, G. Han, M. Thomas, G. Buchanan, H.I. Scher, C.C. Nelson, N.M. Greenberg, L.M. Butler, and W.D. Tilley, A gene signature identified using a mouse model of androgen receptor-dependent prostate cancer predicts biochemical relapse in human disease. *Int J Cancer*, 2012. 131(3): p. 662-72.
13. Schauwaers, K., K. De Gendt, P.T. Saunders, N. Atanassova, A. Haelens, L. Callewaert, U. Moehren, J.V. Swinnen, G. Verhoeven, G. Verrijdt, and F. Claessens, Loss of androgen receptor binding to selective androgen response elements causes a reproductive phenotype in a knockin mouse model. *Proc Natl Acad Sci U S A*, 2007. 104(12): p. 4961-6.
14. Gerber, A.N., K. Masuno, and M.I. Diamond, Discovery of selective glucocorticoid receptor modulators by multiplexed reporter screening. *Proc Natl Acad Sci U S A*, 2009. 106(12): p. 4929-34.
15. Nitiss, J.L., Targeting DNA topoisomerase II in cancer chemotherapy. *Nat Rev Cancer*, 2009. 9(5): p. 338-50.
16. Gewirtz, D.A., A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem Pharmacol*, 1999. 57(7): p. 727-41.
17. Pang, B., X. Qiao, L. Janssen, A. Velds, T. Groothuis, R. Kerkhoven, M. Nieuwland, H. Ova, S. Rottenberg, O. van Tellingen, J. Janssen, P. Huijgens, W. Zwart, and J. Neefjes, Drug-induced

- histone eviction from open chromatin contributes to the chemotherapeutic effects of doxorubicin. *Nat Commun*, 2013. 4: p. 1908.
18. Yang, F., C.J. Kemp, and S. Henikoff, Doxorubicin enhances nucleosome turnover around promoters. *Curr Biol*, 2013. 23(9): p. 782-7.
 19. Gregory, C.W., K.G. Hamil, D. Kim, S.H. Hall, T.G. Pretlow, J.L. Mohler, and F.S. French, Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. *Cancer Res*, 1998. 58(24): p. 5718-24.
 20. van den Heuvel, S. and E. Harlow, Distinct roles for cyclin-dependent kinases in cell cycle control. *Science*, 1993. 262(5142): p. 2050-4.
 21. Chen, S., Y. Xu, X. Yuan, G.J. Bubley, and S.P. Balk, Androgen receptor phosphorylation and stabilization in prostate cancer by cyclin-dependent kinase 1. *Proc Natl Acad Sci U S A*, 2006. 103(43): p. 15969-74.
 22. Warfel, N.A. and W.S. El-Deiry, p21WAF1 and tumourigenesis: 20 years after. *Curr Opin Oncol*, 2013. 25(1): p. 52-8.